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*J Immunol* 2005; 175:4777-4782; doi: 10.4049/jimmunol.175.7.4777
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Induction of Apoptosis by the Hydrocarbon Oil Pristane: Implications for Pristane-Induced Lupus

Nicola Calvani,* Roberto Caricchio,† Marco Tucci,* Eric S. Sobel,‡ Franco Silvestris,* Paola Tartaglia,* and Hanno B. Richards2‡

Intraperitoneal injection of the hydrocarbon oil pristane into normal mice leads to a lupus-like autoimmune syndrome. Although advances in defining the roles of cellular and humoral mediators involved in this syndrome have been made, the mechanisms that initiate a break in tolerance leading to autoimmunity remain unknown. We describe in this study that pristane induces apoptosis both in vivo and in vitro. Pristane arrests cell growth and induces cell death by apoptosis via the mitochondrial pathway of caspase activation in a dose-dependent manner. Nuclear autoantigens created by pristane-induced apoptosis of lymphoid cells within the peritoneal cavity in the setting of a profoundly altered cytokine milieu may be the initiating event in the development of autoimmunity in this syndrome. These findings suggest that apoptosis may be a critical initial event in the pathogenesis of pristane-induced lupus and are of potential relevance for human systemic lupus erythematosus. The Journal of Immunology, 2005, 175:4777–4782.

Because apoptosis has emerged in recent years to explain how self-Ags might become available to a self-primed immune system (7), the aim of this study was to examine the potential role of pristane in the initiation of apoptosis in lymphoid cell lines and in murine peritoneal exudate cells. We report in this study that pristane induces programmed cell death both in vitro and in vivo. We speculate that apoptosis brought about by pristane provides the autoantigen substrate necessary for a break in tolerance, ultimately leading to the development of lupus-like autoimmunity.

Materials and Methods

Cells and culture conditions

BWS147 (murine), Jurkat (human T cell lymphoma), and U266 (human myeloma) cells were obtained from American Type Culture Collection. Freshly isolated peritoneal cells from mice were used in some experiments. Cells were grown in DMEM supplemented with 10% FBS under standard cell culture conditions. Due to its insolubility in aqueous medium, pristane (Sigma-Aldrich) was added as a suspension of inclusion complexes with β-cyclodextrin (β-CyD; Sigma-Aldrich) as previously described (6).

Treatment of mice

Female BALB/cJ, B6, B6 lpr/lpr (The Jackson Laboratory), and BALB/cAn (National Cancer Institute) mice housed under specific pathogen-free conditions, aged 6–8 mo, were used to analyze pristane-induced apoptosis in vivo in most experiments. For the experiments to characterize specific cell populations (as shown in Fig. 5), four 12-wk-old B6 mice housed under conventional conditions were used. Animals were injected once i.p. with either 0.5 ml of pristane or PBS. After 48 h, peritoneal cells were collected by lavage and depleted of RBC by lysis.

FACS analysis

Aptoptosis was assessed and quantified by staining for annexin V-PE, 7-aminoactinomycin (7AAD), and rabbit mAb anti-active caspase 3-FITC according to the manufacturer’s protocols (BD Pharmingen). Peritoneal cells were stained using rat anti-mouse mAbs. For T cell staining, the following combination was used: CD90.2-FITC, annexin V-PE, 7AAD, CD4-B/Av-Pacific Blue, CD3-allophycocyanin, and B220-allophycocyanin-Cy7. For B cell staining, annexin V-PE, 7AAD, CD5-B/V-Ap-Pacific Blue, IgM-allophycocyanin, and B220-allophycocyanin-Cy7 were used. For dendritic cells (DCs) and monocytes, I-A(b)-FITC, annexin V-PE, 7AAD, CD11c/B/Ap-Pacific Blue, CD11b-allophycocyanin, and B220-allophycocyanin-Cy7 were used. All the reagents were purchased from BD Pharmingen, with the exception of the apoptosis kit (annexin and 7AAD) and avidin-Pacific Blue (Molecular Probes). Samples were analyzed with a

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0022-1767/05/$02.00

Received for publication September 24, 2004. Accepted for publication July 25, 2005.

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1 This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Grant NIDDK KO8DK02890-02 (to H.B.R.) and Arthritis Investigator Award (to R.C.).

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; 7AAD, 7-aminoactinomycin; β-CyD, β-cyclodextrin; DC, dendritic cell; Fas-L, Fas ligand; PS, phosphatidylinerine.
DakoCytomation Cyan flow cytometer. Each analysis included a total of at least 10,000 events.

Detection of mRNA expression for Fas and Fas ligand (Fas-L)

Total RNA was isolated, reverse transcribed, and amplified using the ThermoScript RT-PCR System according to the instructions of the manufacturer (Invitrogen Life Technologies). Amplification conditions were 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. Thirty cycles with a final extension of 10 min at 72°C were performed in a thermocycler (MJ Research). β-Actin served as a control. Amplification products were analyzed on agarose gels by ethidium bromide staining. Primer sequences for mouse Abs (overlaid histograms in histograms. Cells stained with isotype controls were used as negative control. After 48 h, the percentage of apoptotic cells (annexin V+/7AAD−, active caspase 3+, and low forward/high side scatter signals) was markedly higher in pristane-treated cells than in control cells (no treatment, β-CyD alone, and n-hexadecane/β-CyD).

Inhibition of caspase activity

Caspase 8 and 9 activities were inhibited by treating the cells for 1 h with Ac-ESMD-CHO (Alexis) or Ac-LHD-CHO (Alexis), respectively. Inhibitors were dissolved in DMSO and added at a final concentration of 20 μM. Thereafter, 200 μM pristane was added to the culture medium for 36 h. Cells were then harvested and evaluated for apoptosis by annexin V labeling. Control experiments used untreated cells as well as cells incubated with DMSO alone.

Statistical analysis

Difference between groups were determined by the Mann-Whitney U test.

Results

Pristane induces apoptosis in lymphoid cell lines in a time- and dose-dependent manner

BW5147 cells were incubated with various concentrations of pristane (up to 400 μM) for up to 48 h. After different time intervals, cells were harvested and stained with annexin V-PE and 7AAD. One of the earlier events during cell death is externalization of membrane phospholipid phosphatidylserine (PS) (9). Annexin V is a phospholipid-binding protein with a high affinity for PS and binds to cells with exposed PS. 7AAD allows for differentiation between early apoptotic cells that stain positively for annexin V but negatively for 7AAD (membrane integrity still present) and nonviable cells that are positive for both annexin V and 7AAD (10). The number of early apoptotic cells (annexin V+/7AAD−) progressively increased between 24 and 48 h of pristane treatment, becoming significantly higher by 48 h compared with untreated control cells (60 ± 1.3% vs 6.2 ± 1%; p < 0.05; Figs. 1 and 2A, pristane vs no treatment). Finally, markedly higher levels of active caspase 3 were found after 48 h in pristane-treated cells compared with untreated cells (46 ± 5.9 vs 6 ± 1.3%; p < 0.05; Fig. 2B, pristane vs no treatment). Finally,
changes in the morphology of apoptotic cells were monitored as a change in light-scattering properties (12). After pristane treatment, cells shifted toward a lower forward (reduction in size) and higher side scatter (increase in granularity), a pattern typical for apoptotic cells (Fig. 2C, pristane vs no treatment). To control for any potential effect of β-CyD, we also incubated cells with β-CyD alone (400 μM). We found no difference between cells treated with β-CyD alone compared with untreated cells. To examine a possible effect of a mineral oil contained within inclusion complexes between β-CyD, we incubated cells with mineral oil and another alkane, n-hexadecane. Neither mineral oil (data not shown) nor n-hexadecane complexed with β-CyD induced apoptosis (Fig. 2). Pristane also induced apoptosis in Jurkat and U266 cells, albeit at slightly lower levels (data not shown). We also examined the dose-response relationship for the induction of apoptosis by pristane. Fig. 3A shows that concentrations of 100 μM or more were required to increase the percentage of annexin V+/7AAD− cells significantly (p < 0.05) compared with no treatment.

**FIGURE 3.** A, Dose-response curve. Apoptosis in BW5147 is shown as the mean percentage (histograms) ± SD (bars) of annexin V+/7AAD− cells after 48 h of treatment with various doses of pristane (mean of 10 individual experiments). The dashed line shows the mean value for untreated controls. *, p < 0.05 vs untreated controls. B, Pristane administration ex vivo is associated with increased apoptosis of peritoneal cells. Freshly harvested pooled peritoneal cells from 6- to 8-mo-old BALB/c J mice were cultured in complete DMEM alone (untreated) or with pristane 200 μM/β-CyD for 48 h. The percentage of viable cells defined as annexin V−/7AAD− is shown in for each time point; the results presented are the mean of three separate experiments. C, Freshly harvested pooled peritoneal cells from BALB/c J mice were cultured in complete DMEM alone (untreated) or with pristane (200 μM/β-CyD) for 6 h. The percentage of annexin V+/7AAD−, annexin V−/7AAD+, and active caspase 3+ cells are shown; the results presented are the mean of three separate experiments. D, Pristane administration in vivo is associated with increased apoptosis of peritoneal cells. Peritoneal cells from 6- to 8-mo-old mice were obtained 48 h after either pristane or PBS treatment and are shown as the mean percentage (histograms) ± SD (bars) of annexin V−/7AAD− (B6 and BALB/cAn) and active caspase 3+ (BALB/cJ) cells.

**Pristane treatment is associated with apoptosis of murine peritoneal cells both in vitro and in vivo**

For the induction of a lupus-like syndrome, a single dose of 0.5 ml of pristane (2 mM) was injected i.p. Although the oil was taken up by tissue relatively rapidly, pristane could still be found in significant quantities in the peritoneal fluid for months. Peritoneal cells were thus exposed to pristane over a prolonged period of time and may therefore be subject to its apoptotic properties. To substantiate this premise in an ex vivo approach, fresh cells collected from the peritoneal cavity of normal mice were cultured with pristane (200 μM) for different intervals of time. Not surprisingly, after 48 h, viable cells (annexin V−PE+/7AAD−) were dramatically reduced with pristane treatment (Fig. 3B), and we found mostly dead cells that stained with both annexin V-PE and 7AAD (not shown). However, the presence of a distinct annexin V−PE+/7AAD− subpopulation and activation of caspase 3 suggested that peritoneal cells had undergone cell death by apoptosis, rather than necrosis secondary to pristane treatment (not shown). Furthermore, time-course experiments revealed that these cells underwent apoptosis by 6 h of pristane treatment (Fig. 3C).

Next, we investigated the effect of pristane treatment in vivo. Freshly isolated peritoneal cells from several common strains of mice were obtained 48 h after treatment with either PBS or pristane (without β-CyD). As shown in Fig. 3D, pristane induced apoptosis, as shown by both annexin V and active caspase 3 staining. In a subsequent experiment, 6- to 8-mo-old B6 mice were injected with either pristane (n = 7) or PBS (n = 7). After 48 h, the number of apoptotic cells in the peritoneal lavage was significantly higher in pristane-treated mice than in control mice, as determined by caspase 3 cleavage (18.2 ± 1.2 vs 2.7 ± 0.9%; p < 0.05; Fig. 4A) and annexin V binding (Fig. 4B). To further define which cell populations were affected by pristane, pooled peritoneal cells from 12-wk-old B6 mice 48 h after treatment with either pristane or PBS were analyzed by FACS. As shown in Table I both
Table I. Peritoneal cell type affected by pristane

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>T Cells</th>
<th>B Cells</th>
<th>Monocytes</th>
<th>Dendritic Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3⁺</td>
<td>B220⁺/IgM⁺</td>
<td>CD11b⁺/CD11c⁻</td>
<td>CD11c⁺/β-A(b)⁺</td>
</tr>
<tr>
<td>Annexin V⁺/7AAD⁻</td>
<td>5.2%</td>
<td>15.9%</td>
<td>14.2%</td>
<td>23.7%</td>
</tr>
<tr>
<td></td>
<td>CD4⁺</td>
<td>CD8⁺</td>
<td>I-A(b)⁺</td>
<td>CD11b⁻</td>
</tr>
<tr>
<td></td>
<td>3.9%</td>
<td></td>
<td>1.3%</td>
<td>25.5%</td>
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</tbody>
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a Percent increase in apoptosis in pooled peritoneal cells from pristane-treated B6 mice above background (PBS-treated mice) 48 h after treatment.

Discussion

To examine the potential mechanisms by which a lupus-like syndrome is induced in normal mice by pristane, we investigated the potential of this hydrocarbon oil to induce apoptosis. To our knowledge this is the first report that pristane induces cell death by apoptosis. To substantiate our findings, we made use of several different techniques for the detection of programmed cell death both in vivo and in vitro: detection of annexin V binding to exposed PS (10), measurement of caspase 3 activation (11), cytochrome c release (13), and evaluation of changes in cell morphology (12). We demonstrate in this study that pristane is a powerful inducer of apoptosis in cultured lymphoid cells, such as BW5147, Jurkat, and U266, and in freshly isolated murine peritoneal cells. We confirm that pristane-induced apoptosis, in our in vitro experiments, is not due to β-CyD alone or a β-CyD emulsion containing another hydrocarbon oil, such as hexadecane, because no apoptosis was induced under either of these culture conditions (Fig. 2).

We report that pristane induces apoptosis in murine peritoneal cells in vivo, thus providing a potential mechanism by which a break in self tolerance may occur in pristane-induced lupus. In particular, we hypothesize that autoantigens exposed through apoptosis induced by pristane and exposed to APCs in the setting of conventional B cells (B2) and B1 cells were more sensitive to pristane-induced apoptosis (annexin V⁺/7AAD⁻) than either CD4⁺ or CD8⁺ T cells. Neither activated (I-A(b)⁺) nor nonactivated (I-A(b)⁻) peritoneal monocytes (CD11b⁺) appeared to be affected by pristane (Table I), whereas markedly increased apoptosis was observed in both myeloid (CD11c⁺/CD11b⁺) and lymphoid (CD11c⁺/CD11b⁻) DCs (Table I). Interestingly a relative accumulation of DCs was observed in pristane-treated mice, with an increase in CD11c⁺/CD11b⁻ cells from 5.1 to 24.3% and in CD11c⁺/CD11b⁻ cells from 1.7 to 5.5% (Fig. 5).

Pristane induces apoptosis via the mitochondrial pathway

In characterizing the molecular events leading to apoptosis through pristane exposure, we found that mRNA transcription for Fas and Fas-L were up-regulated in peritoneal cells from pristane-treated mice compared with those from PBS-treated mice (Fig. 6A). Results were analogous to those observed in BW5147 cells after pristane administration in vitro (Fig. 6A). However, cells lacking a functional Fas pathway, such as those from B6 lpr/lpr mutant mice, were also sensitive to pristane-induced apoptosis in vitro (data not shown) and in vivo (Fig. 6B), suggesting that apoptosis brought about by pristane could occur independently of Fas. Thus, we examined whether the mitochondrial pathway was involved in pristane-induced apoptosis. We detected the release of cytochrome c in BW5147 cells induced to undergo apoptosis by pristane (Fig. 7, A and B), suggesting that the mitochondrial pathway is involved in pristane-induced apoptosis. To further explore the importance of the mitochondrial pathway in pristane-induced apoptosis we conducted experiments to specifically block caspases 8 and 9. As shown in Fig. 7C, pristane-induced apoptosis in BW5147 cells was effectively blocked by the addition of a specific inhibitor of caspase 9 (Ac-LHD-CHO). In contrast, apoptosis was not affected by the addition of a specific inhibitor of caspase 8 (ESMD-CHO; Fig. 7C).

**FIGURE 5.** FACS plots showing the accumulation of DCs in the peritoneal cavity of mice treated with pristane compared with that in mice given PBS. Depicted is the profile of lymphoid and myeloid DCs after gating on class II⁺ events: PBS vs pristane lymphoid DCs, 1.7 vs 5.5%; PBS vs pristane myeloid DCs, 5.1 vs 24.3%.

**FIGURE 6.** A. RNA was extracted from BW5147 after either no treatment or treatment with pristane for 48 h and analyzed for Fas and Fas-L expression by RT-PCR. To examine in vivo effects of pristane on Fas/Fas-L, RNA was extracted from freshly isolated peritoneal cells from BALB/cJ mice 48 h after treatment with either PBS (n = 4) or pristane (n = 4) and analyzed by RT-PCR. Results for each individual mouse are shown. B. Pristane induces apoptosis in B6 lpr/lpr mice in vivo. The representative FACS diagrams show annexin V/7AAD staining in peritoneal cells freshly isolated from B6 lpr/lpr mice 48 h after pristane (n = 3) vs PBS (n = 3) treatment.
In untreated BW5147 cells, cytochrome c is contained within the mitochondrial compartment, as demonstrated by the punctate staining in A. In contrast, after pristane treatment (200 µM for 18 h), cells released cytochrome c by showing diffuse cytoplasmic and nuclear staining, as shown in B. C. Pristane-induced apoptosis in BW5147 was effectively blocked by the addition of a specific inhibitor of caspase 9 (Ac-LHD-CHO). However, apoptosis was not affected by the addition of a specific inhibitor of caspase 8 (ESMD-CHO). The bars represent the mean percentage and SD of apoptosis was not affected by the addition of a specific inhibitor of caspase 9 (Ac-LHD-CHO). However, in experiments studying pristane-treated B6 lpr mice, it has been reported that pristane exposure and defective Fas signaling act on different pathways of autoantibody formation, probably as a consequence of different cytokine requirements (25). Accordingly, our data indicate that pristane-induced apoptosis can occur in the absence of Fas and appears to involve the mitochondrial pathway of caspase activation. However, the early events following the exposure of cells to pristane finally leading to cytochrome c release remained to be defined, and it is unclear whether pristane mediates its effects through a cellular receptor. In studies using nuclear magnetic resonance imaging of pristane uptake into lipid bilayers, it has been shown that pristane localizes to the hydrophobic compartment of the cell membrane (26). It is therefore conceivable that pristane may induce its early apoptotic effects by an indirect, passive, receptor-mediated mechanism, causing changes in the biophysical properties of the membrane bilayers. However, one would expect that other alkanes, such as n-hexadecane, should effect similar cellular changes as pristane, but it did not. The dose-response curve of induction of apoptosis by pristane is sigmoidal and saturable, with a peak at ~300 µM (Fig. 3), which is akin to many receptor-mediated events and argues for the potential existence of a receptor for this hydrocarbon oil.

In conclusion, we present evidence that pristane arrests cell growth and induces cell death by apoptosis in lymphoid cell lines as well as in murine peritoneal cells both in vitro and in vivo. These findings present a potential explanation for the mechanism by which pristane induces autoimmunity. We speculate that uptake of autoantigens provided by sustained apoptosis in the setting of an inflammatory milieu leads to a break in self tolerance and ultimately autoimmunity.

Disclosures
The authors have no financial conflict of interest.

References

FIGURE 7. Pristane induces apoptosis via the mitochondrial pathway. In untreated BW5147 cells, cytochrome c is contained within the mitochondrial compartment, as demonstrated by the punctate staining in A. In contrast, after pristane treatment (200 µM for 18 h), cells released cytochrome c by showing diffuse cytoplasmic and nuclear staining, as shown in B. C. Pristane-induced apoptosis in BW5147 was effectively blocked by the addition of a specific inhibitor of caspase 9 (Ac-LHD-CHO). However, apoptosis was not affected by the addition of a specific inhibitor of caspase 8 (ESMD-CHO). The bars represent the mean percentage and SD of apoptosis was not affected by the addition of a specific inhibitor of caspase 9 (Ac-LHD-CHO). However, in experiments studying pristane-treated B6 lpr mice, it has been reported that pristane exposure and defective Fas signaling act on different pathways of autoantibody formation, probably as a consequence of different cytokine requirements (25). Accordingly, our data indicate that pristane-induced apoptosis can occur in the absence of Fas and appears to involve the mitochondrial pathway of caspase activation. However, the early events following the exposure of cells to pristane finally leading to cytochrome c release remained to be defined, and it is unclear whether pristane mediates its effects through a cellular receptor. In studies using nuclear magnetic resonance imaging of pristane uptake into lipid bilayers, it has been shown that pristane localizes to the hydrophobic compartment of the cell membrane (26). It is therefore conceivable that pristane may induce its early apoptotic effects by an indirect, passive, nonreceptor-mediated mechanism, causing changes in the biophysical properties of the membrane bilayers. However, one would expect that other alkanes, such as n-hexadecane, should effect similar cellular changes as pristane, but it did not. The dose-response curve of induction of apoptosis by pristane is sigmoidal and saturable, with a peak at ~300 µM (Fig. 3), which is akin to many receptor-mediated events and argues for the potential existence of a receptor for this hydrocarbon oil.

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